**USDA, NIFA SCRI HLB Citrus Disease Research and Extension Program**
**2016 Awards (cycle 2) and Project Overview**

**Source: Current Research Information System**

**http://cris.nifa.usda.gov/cgi-bin/starfinder/0?path=fastlink1.txt&id=anon&pass=&search=(GC=CDRE)&format=WEBTITLESGIY .**

**ACCESSION NO:** 1008978 **SUBFILE:** CRIS
**PROJ NO:** CA-R-PPA-5119-CG **AGENCY:** NIFA CALB
**PROJ TYPE:** OTHER GRANTS **PROJ STATUS:** NEW
**CONTRACT/GRANT/AGREEMENT NO:** 2016-70016-24833 **PROPOSAL NO:** 2015-10731
**START:** 01 FEB 2016 **TERM:** 31 JAN 2021
**GRANT AMT:** $3,990,772 **GRANT YR:** 2016
**AWARD TOTAL:** $3,990,772
**INITIAL AWARD YEAR:** 2016

**INVESTIGATOR:** Ma, W.

**PERFORMING INSTITUTION:**
UNIVERSITY OF CALIFORNIA, RIVERSIDE
RIVERSIDE, CALIFORNIA 92521

***EFFECTOROMICS OF THE HUANGLONGBING (HLB)-ASSOCIATED PATHOGEN***

**NON-TECHNICAL SUMMARY:** Huanglongbing (HLB) has caused unprecedented crisis to the citrus industry worldwide. In the US, HLB is associated with the phloem-colonizing, insect-transmitted bacterium Candidatus Liberibacter asiaticus (Las). Since no cures for HLB are available and true resistance in citrus has not been found, methodologies that effectively detect Las in large-scale set-ups and the development of resistant citrus varieties are urgently needed for a long-term solution.One of the most important virulence mechanisms utilized by bacterial pathogens is manipulation of host immunity and physiology through the function of secreted effector proteins. Las possesses the Sec secretion system, through which a variety of Sec-delivered effectors (SDEs) could be secreted into the citrus phloem. Research findings from our research team and other laboratories strongly suggest that SDEs are promising detection markers for robust HLB diagnosis and excellent molecular probes to identify key components required for HLB development.In this project, we will systematically characterize SDEs produced by Las isolated from HLB-infected citrus in the three major producing states, FL, CA and TX. A "core" (likely essential) SDEs produced by all Las isolates will be identified and further used to develop antibody cocktails for HLB detection. Robust HLB detection is essential for the timely removal of HLB-infected trees in order to: 1) prevent the spread of HLB in California; 2) implement therapies and nutritional management programs to extend the productive life of trees before they decline in Texas; and 3) reinforce the replanting effort in Florida. Furthermore, the core set of SDEs will be investigated to understand the molecular basis of their virulence functions. In particular, we will identify the citrus targets of these SDEs and then generate genome-edited citrus to achieve enhanced resistance to HLB. The availability of HLB-resistant citrus would represent a major milestone on combating HLB. Therefore, the outcome of this project will benefit citrus production and profitability over the long term.

**OBJECTIVES:** This Standard Research and Extension Project (SREP) directly addresses critical stakeholder needs represented by two of the four priority areas identified by the Citrus Disease Sub-committee, i.e. "Development of methodologies that allow for the early detection of Las" and "Development of rootstocks resistant to, or tolerant of, Las".The goal of this project is to systematically analyze the Sec-delivered effectors (SDEs) from a variety of Las isolates in different citrus growing areas using genome sequence analysis, expression profiling, and host target characterization. Using this knowledge, we will: 1) develop antibody cocktail-based HLB diagnosis methods that directly detect Las; 2) generate HLB resistant citrus by modifying the citrus targets of SDEs using the recently developed genome-editing approach. Facilitated by vigorous extension and outreach activities, this project will benefit the development of integrative management program for HLB in a sustainable manner.The specific objectives are:1. Systematic analysis of Sec-delivered effectors from various Las isolates;2. Antibody development targeting the core SDEs for HLB detection;3. Identification of SDEs that contribute to HLB pathogenesis;4. Development of genome-edited citrus with HLB resistance;5. Sociological analysis of consumer responses to genome-edited citrus;6. Extension and outreach.

**APPROACH:** Objective 1. Systematic analysis of Sec-delivered effectors from various Las isolatesWe will obtain full genome sequences of Las isolated from Texas, Florida and California using next-generation sequencing including Illumina and PacBio. Potential Sec-delivered effectors (SDEs) will be bioinformatically predicted from the genomes. The expression of SDEs will be determined from HLB-infected trees in the field and using greenhouse experiments.Objective 2. Antibody development targeting the core SDEs for HLB detectionWe will screen a library of synthetic, monoclonal antibodies for those that specifically bind to individual "core" SDEs with high affinity. These antibodies will be used as a cocktail for HLB detection by direct tissue imprint assay and enzyme-linked immunosorbent assay (ELISA).Objective 3. Identification of SDEs that contribute to HLB pathogenesisWe will characterize the impact of SDEs on citrus development and immunity using transgenic citrus expressing individual "core" SDEs. The transgenic citrus plants will be examined for susceptibility to Las infection and monitored for developmental phenotypes reminiscent to HLB symptoms.Objective 4. Development of genome-edited citrus with HLB resistanceWe will: 1) identify targets of SDEs using yeast two-hybrid screening and co-immunoprecipitation followed by mass spectrometry; 2) develop genome-edited citrus plants that interrupt SDE targets using a CRISPR/Cas9 system, which enables simultaneous manipulation of multiple targets; 3) examine HLB resistance of the genome-edited citrus. Note: genome-editing does not involve introducing foreign genes into citrus; genome-edited crops are not considered "GMO" and therefore expected to be better accepted by customers.Objective 5. Sociological analysis of consumer responses to genome-edited citrusWe will investigate how the use of biotechnology and the source of genetic modification influence consumer reactions to citrus products from genome-edited plants when the motivation for the use of biotechnology is to preserve a crop and no external genes are used.Objective 6. Extension and outreachWe will collaborate with communication strategists to recommend antibody-based HLB detection methods and to introduce the concepts and uses of genome-edited citrus through workshops, field day events, grower meetings, and websites.

**ACCESSION NO:** 1008984 **SUBFILE:** CRIS
**PROJ NO:** FLAW-2015-10490 **AGENCY:** NIFA FLAW
**PROJ TYPE:** OTHER GRANTS **PROJ STATUS:** NEW
**CONTRACT/GRANT/AGREEMENT NO:** 2016-70016-24828 **PROPOSAL NO:** 2015-10490
**START:** 01 FEB 2016 **TERM:** 31 JAN 2018
**GRANT AMT:** $1,975,000 **GRANT YR:** 2016
**AWARD TOTAL:** $1,975,000
**INITIAL AWARD YEAR:** 2016

**INVESTIGATOR:** Santra, S.

**PERFORMING INSTITUTION:**
UNIVERSITY OF CENTRAL FLORIDA
12722 RESEARCH PARKWAY
ORLANDO, FLORIDA 32826

# *NIFA CENTERS OF EXCELLENCE: MULTIFUNCTIONAL SURFACE/SUB-SURFACE/SYSTEMIC THERAPEUTIC (COE:MS3T) TECHNOLOGY FOR HLB MANAGEMENT*

**NON-TECHNICAL SUMMARY:** Huanglongbing (HLB) is one of the most devastating citrus diseases, caused by the phloem-restricted bacterium 'Candidatus Liberibacter asiaticus' (CLas). The Asian Citrus Psyllid (ACP, Diaphorina citri) is an insect that carries CLas in its gut, spreading the disease from tree to tree. HLB has caused serious damage to the Florida citrus industry (with over 95% of commercial groves affected), now threatening to become endemic in Texas and increasing numbers of infected trees found in residential California threatening commercial groves. Today, citrus growers are in a critical need of robust treatment methods to save their orchards, protect their investments and continue to make profits. To meet the urgent need of growers, this multi-state, multi-institutional and trans-disciplinary NIFA-CoE project will focus on a comprehensive HLB management solution that targets both the insect and the bacteria. The long-term goal of this project is to develop an industrially-viable, multifunctional bactericidal technology (MS3T) for delivering foliar spray based products for HLB, and others citrus diseases (a path-forward to sustainable citriculture). The proposed non-phytotoxic MS3T product is formulated with natural clay based film-forming ACP repellent material, which also serves as a delivery system for two potent bactericides (surface/sub-surface restricted and systemic). The project will focus on determining how the formulations perform regarding to their stability, rainfastness, roughness, thickness, composition and residuals using state-of-the-art analytical tools for material science. The formulations will be optimized for prime effect on target HLB bacteria and ACP repellency, satisfactory ability to withstand rainfall, while minimizing toxicity to the plants or negative environmental and human health impacts. Growers will apply the MS3T products using conventional foliar spray methods and existing pesticide application equipment. MS3T has attributes to prevent ACP invasion, eradicate CLas population in ACPs and control bacteria in infected trees. Successful outcome of this project will deliver a HLB management solution to growers which will allow them to make profit from HLB-affected trees through minimizing yield loss, improving fruit quality and reducing application frequency (saving labor and materials cost).

**OBJECTIVES:** The goal of the proposed multifunctional surface/sub-surface/systemic therapeutic (MS3T) technology is to offer a comprehensive HLB management solution that is industrially-viable, affordable to growers, sustainable and has minimal negative impact on the

environment and human health. The project is expected to deliver a line of products customized to meet the needs of citrus industries to prevent ACP invasion, eradicate Candidatus Liberibacter asiaticus (CLas) population, eventually controlling CLas titer in infected trees.The MS3T technology offers powerful attributes, which will enable growers to profit from HLB-affected trees through minimizing yield loss, improving fruit quality and reducing application frequency (saving labor cost). After consulting with growers, agrichemical industry partners, regulatory consultants and extension specialists, the Center of Excellence (CoE) team has identified the following immediate research and outreach objectives to meet the project goal.Research objectives:Objective 1. Develop non-phytotoxic MS3T formulation, characterize for residual and optimize synthesis process for achieving best efficacy at low product cost (Santra, Tetard, Labbe).1.1. MS3T material development and optimization (Santra): We will optimize MS3T composition (relative ratio of active components and inerts) to achieve optimal bactericidal (in vitro, greenhouse and field efficacy), rainfastness, phytotoxicity and ACP repellency, while minimizing negative environmental (residual), and human health (cytotoxicity) impacts (comparable to industry standards such as Kocide® 3000, Nordox 30/30 WG). This optimization process will require a team of material characterization experts and therefore will involve Co-PDs (Tetard, Labbe) with relevant expertise.1.2. MS3T material characterization (Santra, Tetard, Labbe)1.2.1. Characterization of MS3T film (Santra, Tetard): Properties of MS3T (and its components) which includes stability, rainfastness, roughness, thickness, composition and residual will be studied using a battery of material characterization techniques (SEM, SEM-EDS, AFM, Optical, Raman, FT-IR, AAS and Disulfine Blue Assay).1.2.2. Zn-chelate structure, stability and interactions (Labbe, Santra): To expedite the Clay+Zn-chelate and Zn-Chelate control (Santra) optimization, molecular modeling and simulations (computational) work will be developed. Specifically we will focus using the models to: (1) study the structure and stability in aqueous solution, (2) study the structure and stability in planta, and (3) study the interaction of Zn-chelate with lignin and cellulose. This study will help the team to understand the stability, transport and mobility of Zn-Chelate in planta.1.2.3. Antibacterial properties studies (Santra): Preliminary antibacterial properties of MS3T formulations and appropriate controls will be evaluated against several model plant pathogens, X. alfalfae (ATCC 49120); P. syringae (ATCC 19310) and C. michiganensis (ATCC 10202) using the following two assays.1.2.3.1. Bacterial growth Minimum Inhibition Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC) Assay1.2.3.2. CFU assay to determine bacterial killing1.2.4. Rainfastness study (Santra): Rainfastness of the MS3T materials will be evaluated by spray treating citrus seedlings in greenhouse conditions. Treated samples and controls will be analyzed using SEM, AAS, Raman, FT-IR techniques.1.2.5. Phytotoxicity study (Santra): Plant tissue damage (phytotoxicity) of MS3T formulations will be studied in an environmental growth chamber using tomato plants (model plant system for preliminary evaluation). We will use citrus plants only for the optimized formulations, which are ready for field trial.1.2.6. Preliminary cytotoxicity study (Santra): Two relevant eukaryotic cell lines will be used for in vitro screening of contact and inhalational toxicity. Cell viability assays will be performed as a measure of toxicity using the standard MTS assay.Objective 2. Evaluate the efficacy of MS3T formulations against ACP and CLas, and optimize the application rate and schedule for optimal disease control (De La Fuente, Lee, Chumbimuni -Torres, Labbe, Tetard, Johnson, Graham, Santra)2.1. Mode of action and systemic activity evaluation (De La Fuente, Lee, Chumbimuni -Torres, Labbe, Tetard): We will study the interaction of MS3T with plant tissues at the surface, sub-surface and systemic level. Results from this study will allow for optimization of the MS3T formulations and will provide valuable information of residual activity.2.1.1. Surface/Sub-surface probing of MS3T (actives) with FT-IR and confocal Raman imaging and spectroscopy (Tetard): We will track Zn-Chelate movement (from surface/sub-surface to phloem tissue) using a FT-IR/Confocal Raman based integrated imaging and spectroscopy technique.2.1.2. Systemic activity of Zn-chelate2.1.2.1. Effect of Zn-chelate on CLas in planta using a model vascular

channels (Santra, De La Fuente): We will study the effect of Zn-chelate (systemic component of MS3T) on the bacterial communities present in phloem, by mimicking the vascular channels using microfluidic chambers, custom microfabricated devices, where bacteria grow under constant liquid flow.2.1.2.2. Zn-chelate - systemic movement, phloem concentration and half-life (Lee, Chumbimuni-Torres, Santra, Johnson): A reliable chemical sensing tool, capable of tracking systemic activity and, estimating phloem concentration level of Zn-chelate between two successive spray applications will be developed for the assessment of spray rate and timings. Using this tool we will estimate the half-life of Zn-chelate to understand its potential fate in planta (proof-of-concept).2.1.2.3. Measuring residual Zn-chelate in plants (Santra, Labbe, Tetard, Johnson): We will develop and use a near infrared (NIR) sensor capable of detecting the presence of these materials using the IR fingerprints of the individual components to study the potential Zn-chelate and Fixed-Quat residuals in plant tissues (proof-of-concept).2.2. Preliminary greenhouse trials: application timing (Johnson, Santra, Labbe): Greenhouse trees will be used for all preliminary tests of flush related application timing, rainfastness and efficacy of MS3T formulations.2.3. Field trials (Johnson, Graham, Santra): The efficacy of the MS3T formulation will be evaluated at three timing schedules and a control: 1) every 21 days in the rainy season, every 60 days in the dry season 2) every 30 days from first spring flush to last fall flush; 3) as needed based on observation of Kaolin clay on leaves and compared to 4) untreated control. Timing may be adjusted based on Kaolin clay coverage and rain patterns.2.3.1. Field trials-efficacy of MS3T formulations against HLB (and citrus canker) and its impact on ACP: Two age classes of trees will be evaluated to determine the field efficacy of selected MS3T formulations against HLB: 3-yr-old (10-20% HLB incidence) and 5-year-old (80-90% 'Ruby Red' grapefruit blocks).2.3.2. Field trial - Efficacy of MS3T against HLB in orange: A sweet orange field trial will test the same applications as for grapefruit to determine the field efficacy of selected MS3T formulations against HLB.2.3.3 Effect of film-based treatment for pest control: Film based trials for ACP controls carried out under CRDF funding (project # 858). Control potential of ACP with Clay-modified material will be studied within the scope of this project.2.4 Effect of MS3T on Candidatus Liberibacter asiaticus in the ACP gut (Johnson): ACP adults and nymphs (if present) will be collected from treated and untreated trees in the field trial for this study. Psyllid DNA extraction and quantification of CLas by qPCR using standard techniques will be performed.

**APPROACH:** The project will generate samples and experimental data from research activities related to:MS3T formulations and their optimizationSamples and experimental data resulting from the comprehensive characterization of MS3T formulationSamples and experimental data resulting from the characterization of MS3T properties and residues on/in citrus plantsExperimental data from greenhouse and field efficacy studiesThe following data will be collected during the characterization of the samples:Optical, AFM, SEM, SEM-EDX, Raman, infrared and UV/Vis spectra, AAS, electrochemistry sensing,New biosensing tools will be developed for systemic study of metal-chelatesAssays: Disulfine Blue Assay, Bacterial growth Minimum Inhibition Concentration (MIC) and Minimum Biofilm Eradication Concentration (MBEC) Assay, CFU assay, cytotoxicity assay, trypan blue exclusion assayAt least, three sets of data will be acquired for each set of experiments.The data will be analyzed and presented in graphic and numerical form. Graphics will be generated using Origin, PowerPoint and Adobe Creative Suite software. Images that will be generated from SEM, AFM and optical microscopy will be processed using the software available for respective instruments (Zeiss for SEM, and Witec Project Four, Nanoscope and Gwyddion for AFM).Greenhouse and field trials on grapefruit and sweet orange will be performed to determine efficacy and the most effective method and timing of application. Currently used field spray application methods will be used assess the economic sustainability of the treatment. Efficacy will be determined based on

bacterial titer, fruit production (boxes), and quality (size, shape, color, and standard juice quality characters including brix/acid ratio). Samples will also be taken from these trees to determine the systemic movement and residue of the particles using the detection techniques under development in objective 1. Using special microfluidic chamber techniques, developed to study vascular bacterial pathogens, the mode of action of MS3T against Liberibacter and/or related bacteria will be determined.All numerical data will be saved in txt files. Figures will be produced in .eps, .jpg or .tif formats. Documents and presentation will be created using Tex, Word, Portable Document Format (PDF), and PowerPoint.Throughout the project, students will be taught the ethical responsibilities towards data management and sharing. The team of PDs will regularly meet student to discuss experimental design and optimization, data acquisition, data evaluation, numerical calculations to evaluate the progress of the project based on collected data.Progress towards project objectives will be assessed annually at project meetings and as needed during the year with focus group meetings of the PD, co-PDs and students.Data management responsibility:Each co-PD will be responsible for storing the data acquired from the project, under a folder that is accessible to PD.Data developed under the proposed project will be deposited onto a data server at the University of Central Florida's NanoScience Technology Center. Access to these data will be controlled by a username/password scheme for faculty and student working on the proposed project. Physical access to the server is controlled by key entry and is limited to IT staff only. In addition to this a copy will be stored on an additional computer and a third copy will be stored on back-up storage equipment. The team will share data regularly and the data and research plan will be discussed at joint group meetings.Archiving of data and samples:The data server will have a space reserved to preserve the data produced over the duration of the proposed project. The team will maintain their access to the data during and after the completion of the proposed project without time limit. In addition, data will also be archived through backup on external hard drives to preserve data in case of loss of server issues. When students collect data at the labs of collaborators, they will be responsible for storing and archiving that data according to policies outlined above. Samples will be stored at the labs were analyses occurred according to safety regulations. All the samples will be labeled and stored.

**ACCESSION NO:** 1008974 **SUBFILE:** CRIS
**PROJ NO:** FLAW-2015-10479 **AGENCY:** NIFA FLAW
**PROJ TYPE:** OTHER GRANTS **PROJ STATUS:** NEW
**CONTRACT/GRANT/AGREEMENT NO:** 2016-70016-24782 **PROPOSAL NO:** 2015-10479
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**GRANT AMT:** $2,800,000 **GRANT YR:** 2016
**AWARD TOTAL:** $2,800,000
**INITIAL AWARD YEAR:** 2016

**INVESTIGATOR:** Stelinski, K. S.

**PERFORMING INSTITUTION:**
UNIVERSITY OF FLORIDA
207 GRINTER HALL
GAINESVILLE, FLORIDA 32611

***TARGETING MICROBES TO CONTROL HUANGLONGBING DISEASE OF CITRUS***

**NON-TECHNICAL SUMMARY:** This project proposes to eliminate huanglongbing (HLB) using a novel, gene-based bacterial therapy strategy that targets the HLB pathogen and Asian citrus psyllid symbionts. Specifically, we propose to develop morpholino-based bactericides to reduce pathogen transmission and eliminate infections in existing trees. Peptide phosphorodiamidate morpholino oligomers (PPMOs) are synthetic molecules that mimic DNA and inhibit bacterial gene expression. They have a proven track record of limiting bacterial populations in the treatment of human pathogens. Development of PPMO bactericides to manage HLB offers a significant advantage over traditional insecticide use or antibiotic therapy. These engineered molecules can be delivered specifically to target bacteria based on gene sequence, avoiding the problems of effecting non-target bacteria. Insecticidal application and replanting infected trees are currently the most effective HLB management strategies; however, these options are not economically sustainable and may not be sufficient to protect young trees from infection. Targeting specific bacteria, such as the HLB pathogen, or ACP endosymbionts needed for transmission and insect survival, are tactics that have gained much favor within the scientific community. The goal of the current project is to provide proof-of-concept data supporting the further development and use of PPMO bactericides for HLB managment in large-scale citrus project.The ultimategoal is to develop a "patentable" product that could be employed by growers within the next five years. Significant extension and socio-economic components are built into the project for training purposes to integrate the novel tools for HLB managment into current programs.

**OBJECTIVES:** The primary goal of the proposed research is to develop a novel technology, Morpholino (PPMO)-EGS, which utilizes RNA-based bactericidal agents for management of CLas and ACP. PPMO-EGS technology will be used to inactivate gene expression in the bacterial plant pathogen, Candidatus Liberibacter asiaticus (CLas) and symbionts in the Asian citrus psyllid (ACP). Research directed toward identification of antimicrobial or insecticidal PPMOs will occur during the first phase of the project (years 1-2).The PPMO biochemistry will be carried out at Yale University, but will be evaluated by the PD and Co-PD to test the efficacy of the PPMO-EGS technology in this biological system (bacteria and psyllid). As mentioned earlier, we have developed a system that can grow CLas under a short term culture period which is long enough to evaluate, antibacterial efficacy experiments using the AA medium plus the PPMO product with CLas bacteria isolated from positive citrus plant sap solutions. We have successfully cultured two of the endosymbiotic bacteria isolated from ACP, and have sequences to target a known Wolbcachia. Efficacy will be evaluate PPMO in cultures, and in live ACP.Following critical assessment of results, several PPMO candidates will be selected for further analysis in larger-scale laboratory and glasshouse efficacy experiments using citrus seedlings and ACP. Comparative analysis of candidates via microinjection, bacteria cultures, and feeding assays will be conducted to analyze the effects on Las, psyllid fitness and transmission capacity.Objective 1: Development of morpholino (PPMO)-EGS Technology to disrupt CLas in Citrus plants. CLas gene expression in plants can be specifically inhibited by developing a cell-penetrating peptide-morpholino oligonucleotide conjugate (PPMO), resulting in a bactericidal effect.Hypothesis 1: CLas-infected citrus trees can be "cured" of infection.Objective 2: Development of morpholino (PPMO)-EGS Technology targeting CLas and bacterial symbionts in ACP. CLas and symbiont gene expression can be specifically inhibited in the psyllid by developing a cell-specific PPMO, resulting in a bactericidal effect. Gene-specific antimicrobials can be delivered using methods developed for RNAi-based technologies to reduce CLas infection and transmission in field settings.Hypothesis 2.1 ACP can be "cured" of infection, effectively eliminating pathogen transmission. Hypothesis 2.2 Removal of ACP symbionts will reduce psyllid fitness, eliminating the population of vectors (insecticidal approach).Socio-economic modeling and outreach: Our proposed economic approach is to perform a benefit cost analysis under the standard assumption that growers' objective is to maximize profit (Nicholson

2002). Therefore, we aim to identify and quantify costs and benefits for Florida citrus growers to manage HLB using a gene-based bactericide. HLB is endemic in Florida, where the average percentage of infected acres (trees) in a citrus operation is 90% (80%) (Singerman 2015). The costs and benefits from using a morpholino-based bactericide will be compared to those arising from a baseline alternative defined by standard current practices, and from a third alternative consisting of replanting trees using Citrus Health Management Areas (CHMA). In this way, we will be able to establish which of the alternatives outweighs the others in each case from an economic standpoint. The expected economic benefit from the use of the bactericide to citrus growers is increased profitability as the result of increased yields due to HLB mitigation. During the two-year timespan of this project, efforts will be focused on surveying growers to establish the costs of citrus production under a baseline. We will share our findings with citrus growers throughout the duration of the project by publishing extension articles that will become available in the project's website. We will also present our findings at different industry meetings.The proposed extension approach is to initiate communication with stakeholders regarding synthetic bactericide/biopesticide information and benefits, and education of public and commodity industries through existing university extension. The outreach objectives for this proposal include determining the most effective means to educate urban and grower communities about benefits of harnessing Morpholino-EGS technology as bactericidal agents to reduce Las and ACP. A key component of this project will be the dissemination of the research findings to the Florida and Texas citrus industries where this research will be conducted. Information will also be disseminated to California citrus growers, who are now looking for new tools for ACP and HLB management as the pest and disease spread throughout that state. We will target each of these state citrus industries with our outreach activities since the entire U.S. industry can potentially benefit from the research findings.During this initial phase of the research program, a series of focus groups and one-on-one interviews will be conducted with growers to better understand their concerns about, and knowledge of, non-insecticide food production techniques. In addition, we will ask the industry to participate in an online survey through the Citrus Research and Education Center (CREC) website. The survey will ask growers about their current knowledge of and feelings for management of ACP and HLB. We will also ask growers about their current practices with regard vector and disease management within each state. At the conclusion of the study, those growers who responded to the original study will be contacted to complete a follow up survey.Trade magazine articles are planned during the course of the project to discuss the development of PPMOs as novel bactericidal management tools. We will also gather quarterly at events organized by IFAS extension throughout the season with a member or members of the project team to discuss the progress of the project. Evaluation of research progress will be discussed and grower input regarding adoption of our new technologies will be assessed at these meetings. Throughout the year, at regular intervals, we will post updates about our research progress on the CREC extension website (www.crec.ifas.ufl.edu).

**APPROACH:** Hypothesis 1: CLas-infected citrus trees can be "cured" of infection. Gene targets will be examined and used to develop suitable PPMO products. DNA can be treated with a suitable restriction enzyme, after an examination of the sequence at either ends of the gyrA gene, and isolation of the relevant gyrA fragment pursued. The fragment is cloned into a plasmid in which it will be under the control of a T7 RNA polymerase promoter, transcribed and the RNA used in tests in vitro with RNase P and an EGS designed suitable to show that the EGS technology will work. The region of the conserved sequence of the gene can be easily utilized for any further work, Southern and Northern blots, on the gyrA mRNA isolated from the bacterium after exposure to the appropriate EGS to demonstrate the effectiveness of the technology in terms of cleaving the targeted RNA.The last step of synthesis of the conjugate will be joining the CPP with the MO to produce a PPMO construct. Some minor adjustments inthe conditions of

synthesis have already yielded higher yields, e.g., raising the temperature of the overnight incubation (20 hr) to 60oC, and calibrating with accuracy the concentrations of different chemicals in the reaction. For example, the concentration of various chemicals used in the linking reaction has to be normalized to the amount of MO in the reaction. The concentration of N, N diiso-propylethylamine was calculated accurately (4.5 equivalents per equivalent of the peptide) in lieu of the prescription to use two drops from a syringe. Aside from delivery of the PPMO products into trees and ACP, the laboratory at Yale will provide sufficient material to carry out all the experiments mentioned here.The amount of PPMO, several grams, needed to do a realistic test of this agent with respect to the infection of full sized citrus plants infected with CLas would stretch the capabilities of any research laboratory. However, quick assays of the effect of the PPMO on the specific mRNA from CLas in our AA medium bioassay, along with already developed bioassays for evaluating the movement and efficacy to eliminate CLas from infected citrus seedlings can be done (Hunter USDA). The medium bioassay is conducted in 96 well plates and for those assays synthesis of conjugates can be easily produced. The PPMO (5 to15 µM final concentration) can be added to 50 µl of sap and the number of bacteria assayed after an amount of time to be determined by the growth characteristics of CLas under these conditions. Validation of PPMO efficacy in Planta. Citrus cuttings or one year-old single-stem Valencia trees (C. sinensis) that are five to eight month post-grafting, and not treated with systemic insecticide will be used for plant assays. rees will be placed in a growth room with CLas-infested ACP for a period of one month until trees are uniformly infected. A subsample of psyllids will be collected following plant inoculations to confirm the CLas infestation rate of the insects. The trees will then be removed and treated with insecticide to eliminate all developmental stages ACP. The trees will be transferred to a greenhouse and maintained in a greenhouse for four months to allow for systemic infection.Prior to treatment, four leaves will be removed from each tree, two from each side of the apex of the tree and two from each side of the base of the canopy, for initial titer (T0) using quantitative real-time polymerase chain reaction (qPCR) assays. Trees will then be treated with candidate PPMOs by either foliar sprays or by root infusion. For root infusions, one lateral root from each plant will be carefully separated, but kept attached to the main root system. This intact root will be used after gently cleaning the lateral root of medium, cutting the root tip, and placing the cut tip into a 50 ml tube containing the sample material. After 24 hours the material will be removed from the tube and replaced by distilled water for the duration of the assay. Seven days post-treatment, four additional leaves will be removed from similar locations as the T0 samples and used to monitor movement of the antimicrobial materials. Data collected will include leaf location for T0 and T1 (right/top, right/bottom, left/top or left/bottom, unless it is determined that this does not provide valuable information). The assay will consist of 25 trees per assay with twenty infected/treated and five healthy/treated. For each assay there also will be five healthy untreated and five infected untreated. Similar assays and monitoring will be completed using foliar spray applications of PPMOs.Detection of CLas will be done using quantitative real-time PCR assays,performed in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using a TaqMan qPCR assay as described by Li et al. (2006) for detection of CLas.Hypothesis 2.1 ACP can be "cured" of infection, effectively eliminating pathogen transmission. Injection and feeding bioassays with psyllids will be conducted by Pelz-Stelinski (Univ. Fla.) to evaluate the efficacy of PPMOs for inhibiting infection/transmission by ACP, and to destroy ACP symbionts, thereby killing ACP at all life stages. Recent investigations suggest that elimination of the ACP symbiont, Candidatus Proftella armatura, should reduce or eliminate CLas transmission.Acquisition assays will be conducted by injecting 20 adult ACP with the PPMO

treatment, and caging on Las-infected 'Valencia' sweet orange plants in mesh enclosures for 2 or 4 week acquisition access periods (AAPs). After each AAP, psyllids will be collected and preserved in 80% ethanol at -20°C for subsequent detection of Las by quantitative real-time PCR (qPCR) following the procedures described below and by Pelz-Stelinski et al. (2010).To evaluate inoculation efficiency following exposure to PPMO's, 50 newly-emerged adult psyllids reared on infected plants will be injected with PPMO treatments and released onto uninfected plant leaves. Successful inoculation of leaves by treated ACP will be compared to inoculation by ACP not exposed to CLas-targeting PPMOs. This experiment will be replicated five times per treatment. After a two-week inoculation access period (IAP), psyllids and leaves will be removed and tested for CLas with qPCR as described by Pelz-Stelinski et al. (2010) and Coy et al. (2014).Hypothesis 2.2 Removal of ACP symbionts will reduce psyllid fitness, eliminating the population of vectors (insecticidal approach). The effect of symbiont-targeting PPMOs on psyllid life history will be evaluated in a series of fitness studies that will assess fecundity, survival, and development. To assess the fecundity of PPMO-exposed insects, individual pairs of 3-day-old male and female psyllids will be held on in Petri dishes for acquisition of symbionts using an artificial feeding method or by direct microinjection of symbiont cultures. Control insects will be fed a symbiont-free (filter-sterilized) sugar solution or injected with saline buffer. Insect pairs will then be transferred to pathogen-free plants with flushing plant tissue for mating and oviposition. The number of eggs produced per pair will be counted and removed every five days under a stereomicroscope. Each treatment will be replicated 15 times per psyllid pair.Development time of psyllid nymphs will be assessed by releasing 200 PPMO-exposed or non-exposed pathogen-free citrus plants. After five days, adults will be removed and the number of eggs per plant counted. Immature and adult progeny will be counted at 3 d intervals until adult emergence. Symbiont infections in parent and adult psyllid offspring will be determined using the qPCR assay described below. Survival of symbiont exposed or unexposed psyllids on healthy citrus plants will be compared by releasing 20 newly emerged adult insects of each gender onto plants.

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**PROJ NO:** FLAW-2015-10491 **AGENCY:** NIFA FLAW
**PROJ TYPE:** OTHER GRANTS **PROJ STATUS:** NEW
**CONTRACT/GRANT/AGREEMENT NO:** 2016-70016-24844 **PROPOSAL NO:** 2015-10491
**START:** 01 FEB 2016 **TERM:** 31 JAN 2020
**GRANT AMT:** $3,999,508 **GRANT YR:** 2016
**AWARD TOTAL:** $3,999,508
**INITIAL AWARD YEAR:** 2016

**INVESTIGATOR:** Gabriel, D. W.

**PERFORMING INSTITUTION:**
UNIVERSITY OF FLORIDA
207 GRINTER HALL
GAINESVILLE, FLORIDA 32611

## *DEVELOPMENT NEW THERAPIES FOR HUANGLONGBING VIA CULTURING CA. LIBERIBACTER ASIATICUS*

**NON-TECHNICAL SUMMARY:** Much progress has been achieved in the control of newly emerging plant diseases by first understanding the various strategies used by the pathogens to cause disease and overcome native plant defenses, and then by interfering with key elements of their life cycles, infection strategies, pathogenicity determinants and/or counter-defenses. Genomic DNA sequencing allows the identification of all of the suspected genes in a pathogen's offensive and defensive arsenals. Functional genomics typically confirms suspected pathogen mechanisms for both offense and defense, and provides a firm basis for testing of disease control strategies in infected plants. With newly emerging pathogens, this often involves identification of key new pathogenicity genes, functional confirmation, and hypothesis testing to develop control measures. When a pathogen is uncultured, Koch's postulates cannot be completed, functional genomics becomes severely impacted, and hypothesis testing is extremely difficult and limited. This has been the case with Huanglongbing (HLB) and its associated agent, Ca. Liberibacter asiaticus (Las). A priority for functional studies is to culture Las, in order to leverage the abundant available genomic information and use it towards developing disease control and/or management methods. Limited progress has been made in attempting to culture Las. Only 2 labs (Davis and De La Fuente) have published substantial lab results indicating partial progress towards the goal. Lack of success has likely been due to multiple reasons, including: 1) missing required metabolites in media tested; 2) missing genes or pathways required for free-living growth in the specific Las strain used; 3) unrecognized toxic substances in some media used; 4) activation of phage lytic cycles genes in the Las genome, 5) a substantially weak Las outer membrane barrier, and 6) missing host gene regulation functions following separation of Las from an intracellular host environment. Any combination of these factors could prevent growth, even when a perfect combination is hit with a particular media formulation. What is perhaps most frustrating is that Liberibacter crescens (Lcr) can be readily cultured, and yet it has only a 20% larger genome than Las (1.5 Mbp for Lcr vs 1.26 Mbp for Las). Lcr is limited in that it may no longer be pathogenic or capable of growth in any host, despite dedicated efforts by several labs to inoculate it into different plants and insects. Koch's postulates have yet to be completed for Las or Lcr. Lcr has nevertheless been used (Gabriel lab) to develop a functional genomics toolkit for 1) Las gene expression; 2) characterization of Las phage gene promoters and key regulators, and 3) early and late gene reporter constructs capable of high throughput screening for small molecules that may affect phage gene expression, either directly or indirectly. The Lcr

functional genomics system allowed identification of the Las prophage peroxidase as a likely critical lysogenic conversion gene that is needed by Las to both suppress citrus defense (peroxide) and citrus defense signaling (Gabriel lab). Las peroxidase is thus a potential target for Las control in citrus. Published comparative gene expression analyses (Gabriel lab) have shown that high levels of phage late gene expression, including lytic genes, occurs in Las-infected periwinkle, less in citrus, and almost none in psyllids. Since phage lytic replication does not occur in psyllids, indicating that all phage genes are under stringent repression in psyllids, but less so in citrus. The Las late gene reporter construct was used in the Lcr system to allow identification of a 27 kDa protein in psyllid extracts that directly binds to the late gene promoter and behaves as a strong repressor (Gabriel lab, unpublished). Since all Florida Las strains examined to date carry similar phage, it may be possible to artificially de-repress the phage lytic cycle and/or individual lytic genes in Las infected psyllids and citrus trees in the field and thereby cure both. This repressor and the promoter it binds to are thus two additional potential targets for Las control in psyllids, and expression of this protein is proposed here to help enable culturing of Las. More recently an additional key Las phage regulatory gene was identified by analysis of Las gene expression constructs in Lcr (Gabriel lab), helping to explain why Las phage particles readily form in periwinkle, but not in citrus, and providing another potential control target. Although Lcr has been genetically manipulated and is tractable for transformation, including knockout and knock-in mutations, Las has yet to be transformed so that not even a single genetically marked Las strain has been produced. Although antibodies have been produced that might be used to trap and concentrate Las (for observational, transformation, and culturing purposes), the specificity and threshold level of detection of the antibodies was not reported. This proposal is to: 1) coordinate simultaneous parallel culturing efforts in 4 labs (Duan, Davis, Killiny and De La Fuente) using a variety of complementary approaches and real-time data and deliverable sharing of what works and what does not, and using different Las sources (insect vs. plant); 2) to leverage what has been learned from functional genomic analyses of Lcr and the Las phage in continuing efforts to use Lcr as a culturable proxy (Gabriel) to engineer Lcr to be more Las-like in terms of its outer membrane to allow a more straightforward approach to obtaining antibodies with high specificity and affinity (Ma), and 3) to develop a phage vector (Jones & Gabriel) and/or conjugation system (Gabriel) so that Las can be genetically marked and engineered to enable growth on artificial media.

**OBJECTIVES:** This is a Standard Research and Extension Project (SREP) focused on culturing Ca. Liberibacter asiaticus (Las), with direct applications expected in Huanglongbing (HLB) control and detection/diagnostics. Despite the enormous efforts that have gone into obtaining 5 complete genomic DNA sequences of the 3 different species of HLB bacteria, not one of the causal species has been cultured, and potential molecular targets for control cannot be functionally validated. Most culturing efforts have focused on nutritional supplements and various media formulations in an attempt to supply presumably missing nutrients, but only to a single Las genotype or clonal group. This may be the wrong approach. One Liberibacter species, L. crescens (Lcr), was readily cultured many years ago using very standard bacterial techniques. Its genome is 20% larger than all other sequenced Liberibacters, and has all genes needed for culture. It is also missing specific phage lytic genes that can kill their bacterial hosts under stress conditions and are found in most Las strains and may limit culture. Unfortunately, the single extant Lcr strain also appears to have lost pathogenicity and so is not useful for citrus or general plant pathogenicity assays. Continued attempts to culture a single Las genotype or clone may be fruitless, and therefore multiple parallel approaches are proposed here, including attempts to culture a phageless Las strain, a new citrus species from Colombia that may be related to Lcr, microfluidic chambers, improved chemical screens and those involving improved monoclonal antibodies, and both chemical and phage therapies for HLB. Objective 1: Concerted, parallel and coordinated efforts towards obtaining viable Las and/or Lca cultures (Castañeda, Davis, De

La Fuente, Duan and Killiny). a. Psyllid hemolymph and citrus phloem sap composition b. Enhanced growth observed using the TPIMS chemical library: Objective 2: Define the influence of physical environment in Las culturability (De La Fuente & Ma). a). Cell surface antigens suitable for antibody generation b). Outer membrane proteins as targeted c). Generation of antibodies d. Antibody evaluations e. Microfluidic chambers. Objective 3: Define the role of chemical signaling and co-factors in culturability of Las and/or Lca (Davis, De La Fuente, Gabriel & Killiny). Objective 4: Develop genetic tools that enable delivery of (missing) candidate growth factor genes identified and identified and partially characterized into Las (Gabriel & Jones) a). Screen multiple sources of Liberibacter spp. for phage particles and for Lcr?infecting phages b). Develop assays for detecting phage binding to and infection of Las c). Develop Lcr/Las-cross-reacting phages as a transduction system for genetic modification of Las d). Use elements of the SC1 and SC2 prophages of Las to develop a cosmid vector/phage transfection system. Objective 5: Outreach (Alabi, Roberts, Vidalakis)

**APPROACH:** Objective 1: Concerted, parallel and coordinated efforts towards obtaining viable Las and/or Lca cultures (Castañeda, Davis, De La Fuente, Duan and Killiny). Published empirical approaches to Las culture and use of diverse nutritional resources will be continued (Davis, De La Fuente). These will be expanded in two additional labs (Duan, Killiny), using microarrays, combinatorial libraries, detailed phloem sap analyses from healthy versus infected citrus, fresh versus spent media, psyllid hemolymph, and honeydew. Data will be shared amongst all laboratories so that continual improvements in media formulations can be made. Inoculum derived from both psyllid (Davis, Killiny and Duan labs) and citrus, including phageless Japanese isolates (De La Fuente) will be tested. Phage lytic cycle inhibitors from psyllids will also be provided (Gabriel).Lca is tentatively expected to be imported on bud-sticks from infected material in Colombia; materials will be collected in Colombia (refer attached letter, Castañeda) and imported by Gabriel. Following grafting to clean citrus, material will be monitored for Lca and curated separately from Las infected materials in the Plant Containment Facility. If Lca can be maintained in citrus, Davis will travel to Gainesville to assist in attempts to culture from this material. Objective 2: Define the influence of physical environment in Las culturability (De La Fuente & Ma). All attempts to culture Las to date have used standard batch systems such as agar plates, test tubes, and flasks. Bacteria can behave much differently under different pressures than when grown in batch cultures. Las grows actively in phloem cells where nutrients are being transported by liquid flow, and pressures are high and fluctuating. The microfuidic chamber currently used in the De La Fuente lab allows experimental variation in growth pressures, temperatures, gas exchange and flow rates. Antibodies are needed in order to coat the microfluidic chambers and both increase initial titer of the inoculum and to observe Las behavior in these chambers. The Ma lab will generate high affinity monoclonal antibodies (MAbs) against two classes of cell surface antigens. Firstly, the variable O-antigen epitopes in the outer membrane structural lipopolysaccharide (LPS) will be used to develop species-specific antibodies. LPS will be extracted from Lcr, and high affinity, Liberibacter-specific antibodies will be screened using a library of synthetic, monoclonal antibodies generated by Dr. Xin Ge's laboratory at U.C., Riverside. Secondly, antigenic outer membrane proteins, such as abundant adhesins, will be identified for antibody development using the same library. MAbs will also be useful to develop non-PCR detection methods, such as ELISA Objective 3: Define the role of chemical signaling and co-factors in culturability of Las and/or Lca (Davis, De La Fuente, Gabriel & Killiny) One of the few examples of successful culturing of Las (Davis) was achieved when Las was co-cultured with another bacterium. Attempts at co-cultivation with Lcr will be made to identify unknown signals using a diffusion 'sandwich' approach in agar plates with

macerated suspensions of infected and non-infected citrus and psyllids. In addition, filtrates from secreted compounds obtained from a metagenomics library; and addition of quorum sensing molecules (Killiny) and siderophores will be evaluated. Direct cultures of Las with Lcr engineered with conditional inhibitors (sucrose sensitivity or temperature sensitive lethal conditional mutations will be attempted. Microfluidic chambers are ideal for direct injection and testing of identified signals or co-factors for Las response (De La Fuente).Objective 4: Develop genetic tools that enable delivery of (missing) candidate growth factor genes identified and identified and partially characterized into Las (Gabriel & Jones) At least one likely necessary growth factor gene of Lcr has been identified (Gabriel lab) that is completely missing from the Las genome. Lcr has proven to be genetically tractable for gene knockouts and gene additions, and two compatible DNA shuttle vectors capable of conjugational transfer and phage packaging (cosmid) and transfection have been proven for use in Lcr (Gabriel lab). The standard functional tools developed for Lcr have not been applied to Las because such techniques are not readily applied to uncultured cells, primarily because of low cell density. Conjugation or phage transfection could overcome these issues, and both will be attempted. If co-cultures are successful (Objective 3), conjugational transfer of a vector carrying the growth factor gene plus marker could be likely achieved using an Lcr strain engineered to die if chemically or genetically induced (CRISPR-Cas9; Yang lab). T4-like phages from S. meliloti have been characterized and some, but not all key Las phage structural phage elements identified (Jones lab). Gabriel & Jones will attempt to create an in vitro Las phage packaging system for genetic manipulation of Las. If successful, such a phage system may be further developed (Gabriel & Yang) into a novel therapeutic agent for targeted killing of Las in infected tissue. Once cultured, Las would need to be reintroduced into psyllids, likely by injection (Killiny, Duan labs), and subsequently into citrus. Objective 5: Outreach (Alabi, Roberts, Vidalakis) The ability to successfully culture Las in vitro will be a huge milestone for the scientific community working on the citrus-HLB-ACP pathosystem. However, it is uncertain if growers and other stakeholders in the citrus industry will fully appreciate the importance of such a breakthrough. Hence, outreach activities will need to be developed and implemented to educate members of the public on the importance/ relevance of the project right from its inception. Even if not considering the different degrees of HLB epidemic in the three leading citrus-producing states, the proposed outreach plan will be implemented such that there is a cohesive message on the promise and outcomes of the project. At the project inception, the focus of the outreach activities will be to educate growers, industry stakeholders and members of the public on the importance of being able to culture Las in vitro. Subsequent activities will then focus on providing periodic project updates and breakthroughs to the stakeholders. Finally, news of successful culturing of Las will be announced via face-to-face, print and electronic media materials to be developed jointly by Vidalakis (California), Roberts (Florida) and Alabi (Texas) in consultation with the PD and other co-PIs. Specific outreach activities planned for the project include:

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**PROJ NO:** MD.W-2015-10475 **AGENCY:** NIFA MD.W
**PROJ TYPE:** OTHER GRANTS **PROJ STATUS:** NEW
**CONTRACT/GRANT/AGREEMENT NO:** 2016-70016-24779 **PROPOSAL NO:** 2015-10475
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**GRANT AMT:** $1,951,763 **GRANT YR:** 2016
**AWARD TOTAL:** $1,951,763
**INITIAL AWARD YEAR:** 2016

**INVESTIGATOR:** Cilia, M.

**PERFORMING INSTITUTION:**
NATIONAL AGRICULTURAL LIBRARY
ABRAHAM LINCOLN BUILDING 10301 BALTIMORE AVENUE
BELTSVILLE, MARYLAND 20705

# *HARNESSING NATURAL VARIATION IN TRANSMISSION OF LIBERIBACTER BY THE ASIAN CITRUS PSYLLID TO DEVELOP NOVEL HLB CONTROL STRATEGIES*

**NON-TECHNICAL SUMMARY:** Huanlongbing (HLB) is the most serious disease of citrus. Candidatus Liberibacter asiaticus (CLas), the pathogen associated with HLB, is spread around the grove by the Asian citrus psyllid, a sap-sucking insect vector. Controlling the insect spread of CLas represents the most promising way to control the disease. However, there is a paucity of information on how CLas is spread by the psyllid and currently no tools for a grower to use to detect CLas in the insect. Current detection methods in insects and in plants rely on qPCR and microscopy, and both methods require a laboratory, a skilled researcher, expensive equipment and costly consumables. Our team will pursue four research objectives and one educational objective to better understand how the psyllid transmits CLas in an effort to develop new transmission blocking tools. Drawing upon a new cutting edge scientific field called synthetic biology, we will also develop a low-tech biosensor that a grower can use to detect CLas in insects in the field, one of the goals of the SCRI program. Undergraduate researchers will be involved in all aspects of the synthetic biology project. Although the research plans within each objective are synergistic, the success of one objective does not depend on the others. We realize our research plans are ambitious but our team is up to the challenge.

**OBJECTIVES:** Huanglongbing (HLB) is a tritrophic disease complex involving citrus host trees, the Asian citrus psyllid (ACP) insect and a phloem-restricted, bacterial pathogen Candidatus Liberibacter asiaticus (CLas). HLB is considered to be the most devastating of all citrus diseases, and there is currently no adequate control strategy. In Florida, an estimated 40-70% of all citrus trees are infected, and HLB effects include production declines (10-20% per year), diminished fruit quality and increased production costs. Some growers have already been forced into bankruptcy. California and Texas have the ACP and isolated reports of HLB, where the spread of HLB is imminent without discovery and implementation of new management practices. Control of ACP-mediated CLas transmission represents a promising, new avenue for HLB control, but there is a paucity of tools available for growers to control HLB from this angle.Our team will pursue four research objectives and one educational objective. Although the research plans within each objective are synergistic, the success of one objective does not depend on the others. We will:1. Discover genetic populations of ACP that

segregate for CLas acquisition and transmission competency.2. Functionally characterize the ACP endosymbiont toxin diaphorin and establish whether the relationship between the ACP endosymbiont Profftella and the ACP is a viable target for ACP control.3. Functionally characterize ACP proteins involved in CLas transmission.4. Develop an ACP-CLas yeast biosensor that can be used by growers5. Enhance cross-disciplinary undergraduate education and research at Cornell through participation in the International Genetically Engineered Machines (iGEM) Competition.The overall, long-term goal of our project is to develop new tools that can be used by citrus growers for CLas-infected ACP monitoring and management, as detailed in Objectives 3 and 4. Two short-term goals of our project that we will achieve during the lifetime of our grant include: to characterize populations of the ACP that vary in their ability to transmit CLas and to produce a yeast biosensor that can readily detect CLas-infected ACP. The ACP populations will be immediately useful for challenging citrus germplasm against HLB and ACP inoculation and feeding. A long-term goal is the distribution of the biosensor technology to growers. A second long-term goal of our proposal is to leverage the knowledge of the molecular basis of CLas transmission by the ACP to produce a genetically modified citrus that will prevent the spread of CLas within a grove. All research will be performed with constant engagement of the stakeholders to provide support and guidance on technology advancement through to commercialization.

**APPROACH:** Objective 1: Colonies of potentially good or poor vectors will be established using an individual adult female ACP paired with a single male collected from various locations. Insects will be determined to be CLas-free to start and CLas transmission efficiencies will be calculated using transmission assays. Efficient and poor transmitting lines will be crossed and their progeny randomly mated to develop populations segregating in CLas transmission ability.Objective 2: Mass spectrometry-based proteomics will be used to identify proteins interacting with the toxin diaphorin. Potential receptors will be functionally validated surface plasmon resonance and other methods. CTV technology will be used for silencing the diaphorin receptor in the ACP.Objective 3: The CTV technology has been established by the Dawson lab previously. Construction of CTV vectors that target ACP proteins involved in valine catabolism, immune system regulation and bacterial entry is currently in progress in the Cilia and Dawson labs, greenhouse tests to follow.Objective 4: Ongoing research in the Cornish?Laboratory has demonstrated the ability to take?advantage of an endogenous G protein-coupled?receptor (GPCR) signaling pathway present in Saccharomyces cerevisiae to drive a lycopene?biosynthetic pathway and directed evolution. These methods may be implemented to engineer?a GPCR to bind a specific peptide biomarker of CLas in infected ACP. These methods will be used to engineer yeast to turn red when mixed with insects or plant tissue harboring CLas, enabling a rapid and clear determination of CLas status by a grower in the field.Objective 5: iGEM undergraduate research teams will be formed at Cornell and Columbia Universities and will assist with achieving objective 4.

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**PROJ NO:** NM.W-2015-10483 **AGENCY:** NIFA NM.W
**PROJ TYPE:** OTHER GRANTS **PROJ STATUS:** NEW
**CONTRACT/GRANT/AGREEMENT NO:** 2016-70016-24781 **PROPOSAL NO:** 2015-10483
**START:** 01 FEB 2016 **TERM:** 31 JAN 2018
**GRANT AMT:** $3,320,000 **GRANT YR:** 2016
**AWARD TOTAL:** $3,320,000
**INITIAL AWARD YEAR:** 2016

**INVESTIGATOR:** Gupta, G.

**PERFORMING INSTITUTION:**
NMC, INC.
4200 W JEMEZ RD
LOS ALAMOS, NEW MEXICO 87544

***DESIGN AND DELIVERY OF THERAPEUTIC PROTEINS FOR HLB PROTECTION***

**NON-TECHNICAL SUMMARY:** So far there is no cure for HLB nor is there any HLB-resistant citrus. Therefore, protection measures are urgently needed to save the infected trees and to protect the healthy ones. In this CAP, we will perform systems level studies to understand the battle between Liberibacter and citrus, i.e., Liberibacter attempting to propagate its lifecycle while citrus is trying to clear Liberibacter by mounting innate immune defense. We will use this knowledge to make the innate immune system of citrus stronger to win the battle against Liberibacter. This will be achieved by designing two classes of citrus or citrus-like proteins, i.e., one that directly targets and clears Liberibacter from the phloem and the other that prevents detrimental Liberibacter proteins from disrupting innate immune defense in citrus. We propose to develop delivery strategies for short-term (6 months), intermediate-term (1-2 years), and long-term protection (3-5 years) protection. With these robust and cost-effective tools, the growers will be able to save the infected trees and protect the healthy ones.

**OBJECTIVES:** GoalsThis CAP focuses on engineering novel innate immunity in citrus via the design and delivery of therapeutic proteins that facilitate rapid clearance of Liberibacter and inhibition of key steps in the development of Huanglongbing (HLB). Unlike small molecule antibiotics and RNAi, the proposed therapy is based on endogenous proteins derived from the citrus innate immune repertoire. The main goals are to develop: (i) a novel strategy for designing therapeutic proteins based on genome-wide studies on Liberibacter-citrus interactions and (ii) transgenic and non-transgenic delivery methods that offer short-, intermediate-, and long-term solutions for Huanglongbing (HLB) protection. This CAP specifically addresses the areas 1 and 4 of the call (page 6 in the call):Bacterial therapy systems that either kill or suppress Candidatus Liberibacter asiaticus (Liberibacter)Development of citrus scions and rootstocks resistant to, or tolerant of, Liberibacter that are suitable for a wide range of growing environments.ObjectivesObjective 1. Identify (a) Liberibacter genes/proteins and (b) citrus genes/proteins as potential targets for therapies to protect against HLB.Objective 2. Design and express HLB-protective proteins that either clear Liberibacter or block disease development.Objective 3. Deliver HLB-protective proteins in planta for short-, intermediate-, and long-term protection.

**APPROACH:** Objective 1 (Target Identification)(Team: Mikeal Roose, UC Riverside; Rakesh Kaundal, UC Riverside; Goutam Gupta, NMC/NMC; Geoffrey Waldo, NMC/NMC; Hau Ngyuen, NMC)For Liberibacter killer proteins, we will first identify the molecular entities on the Liberibacter membrane that can be targeted by specific citrus proteins. Two high priority targets are the outer-membrane proteins (OMPs) and the conserved lipopolysaccharide (LPS) core, which, as we have shown (see below), can be recognized respectively by citrus proteases and LPS-binding proteins (LBP). For the chimeras, lysis domains will be citrus linear or disulfide-bridgedantimicrobial peptides (AMP), which bind and rupture gram-negative bacterial membranes. We will perform structural and computational analyses to prioritize Liberibacter recognition and lysis domains. For the protein inhibitors of HLB, we will first identify the critical protein-protein interactions in Liberibacter-citrus interactions, for example the ones in the signaling pathways involving pathogen-associated molecular pattern triggered (PTI) and effector triggered signaling. PTI and ETI signaling constitute an important part of plant innate immune defense. Specific pathogen proteins (virulence factors and effectors) tend to disrupt one or more steps in the PTI/ETI signaling pathway, and inhibiting this disruption will be an effective strategy for HLB protection.Specific Tasks will include:Task 1a: (i) To analyze Liberibacter genomes to identify the conserved outer-membrane proteins (OMP) and lipopolysaccharides (LPS); (ii) To screen citrus genomes to identify citrus proteases that cleave Liberibacter OMP and LBP that bind LPS; (iii) To analyze citrus genomes to select citrus AMPs that lyse gram-negative bacteria such as Liberibacter.Task 1b: (i) To measure and analyze dual Liberibacter-citrus transcriptome at different stages of infection (early to late) to predict candidate interactions between Liberibacter effectors and citrus innate immune defense proteins; (ii) To validate the predicted protein-protein interaction pairs by a three-body split-GFP reporter assay.Objective 2 (Design and expression of therapeutic proteins for HLB Protection)(Team: Goutam Gupta, NMC/NMC; Hau Nguyen, NMC/NMC; Geoffrey Waldo, NMC/NMC)We will use structure-based algorithms to design Liberibacter killers and HLB-blockers and will express the therapeutic proteins in tobacco BY-2 cell lines.Specific Tasks will include:Task 2a: To design protein chimeras with recognition (e.g., protease and LBP) and lysis (AMP) domains to rapidly clear Liberibacter. We have already designed three chimeras: (i) tobacco Thionin-D4E1 chimera (tobacco Thionin is 70% identical in sequence to citrus Thionin; D4E1 is a citrus-friendly synthetic AMP); (ii) citrus Thionin-LBP1 chimera; and (iii) citrus Thionin-LBP2 (LBP1 and LBP2 are two citrus LBPs). Note that Thionins have both recognition and lysis functions.Task 2b. To design citrus protein mimics that bind and sequester Liberibacter effectors and inhibit their detrimental interaction with citrus proteins involved in plant innate immune defense.Task 2c. To use two-body split-GFP system to monitor expression therapeutic proteins in tobacco BY-2 cell expression system.Objective 3 (Delivery and efficacy testing of the therapeutic proteins)(Team: Ed Stover, USDA-ARS, Ft. Pierce, FL; William Belknap, Beltsville, MD; James Thomson, USDA-ARS, Albany, CA; Siddarame Gowda; University of Florida, Lake Alfred)We will develop short-, intermediate-, and long-term strategies for in planta delivery of the therapeutic proteins.Specific Tasks will include:Task 3a: To design lipid-based nanocapsules coated with citrus tristeza virus (CTV) capsid protein to deliver the therapeutic proteins; To test efficacy by determining Liberibacter level in greenhouse infected trees by qPCR at 0.5, 1, 2, 4, 8, and 12 months after initial delivery of the therapeutic proteins. Tree growth, health, and HLB symptoms will be monitored at 1, 2, 4, 8, and 12 months after the delivery of the therapeutic proteins.Task 3b: (i) To use CTV as a vector to express the therapeutic proteins for protection against HLB; (ii) To graft transmit this vector to express HLB therapeutic proteins in different infected citrus cultivars and monitor the efficacy as described in Task 3a.Task 3c: (i) To develop

tools for more efficient and robust transgene expression (i.e., improved transcriptional control elements for optimal phloem-specific expression, CRISPR-like systems to repress, edit, or overexpress endogenous genes); (ii) To construct transgenic citrus lines expressing already designed Tobacco Thionin-D4E1, Citrus Thionin-LBP1, and Citrus Thionin-LBP2 chimera and test their efficacy in HLB protection. Note that we have already demonstrated that transgenic citrus (Carrizo) expressing Tobacco Thionin-D4E1 is protective against HLB. The other two (i.e., Citrus Thionin-LBP1, and Citrus Thionin-LBP2) chimeras have been designed to show higher efficacy than the Tobacco Thionin-D4E1 chimera.

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**INVESTIGATOR:** Gang, D. R.

**PERFORMING INSTITUTION:**
WASHINGTON STATE UNIVERSITY
240 FRENCH ADMINISTRATION BLDG
PULLMAN, WASHINGTON 99164-0001

***DEVELOPMENT OF IN VITRO BIOFILM AND PLANKTONIC CULTURE OF CA. LIBERIBACTER ASIATICUS: A GAME CHANGE IN HLB RESEARCH***

**NON-TECHNICAL SUMMARY:** The lack of ability to culture "Candidatus Liberibacter asiaticus" (CLas) has precluded the use of genetics and gene transformation approaches to identify gene functions involved in CLas viability or virulence. Those are critical tools that will ultimately be needed in development of a fully integrated strategy to control CLas and eventually develop the capability to stop HLB spread by the Asian Citrus Psyllid (ACP) and the resulting economic devastation to citrus agriculture. This project aims to develop means to culture CLas in vitro using two novel, parallel, complementary and integrated strategies that also integrate proteomics, metabolomics and community sequencing approaches. This will ultimately lead to successful culture of CLas under both planktonic growth conditions and as biofilms.

**OBJECTIVES:** The first major goal of this project is to develop a system to culture, in vitro, "Candidatus Liberibacter asiaticus" (CLas), the causative agent of Citrus Greening Disease, or Huanglongbin (HLB). A second major goal is to then make that system readily available and adopted by the national stakeholders, particularly citrus breeders and other researchers, thereby enabling genetics-based research that will have an enhanced capability to identify means to control the transmission of CLas. A third goal, which will be a natural offshoot of the other goals, is to develop rapid and easy methods to isolate and cultivate new strains of CLas from groves across the nation as well as adapting or mutating strains of CLas within regions of outbreaks or reintroductions that may occur even if means to control CLas are indeed developed. Culturing of new strains like that will be critical long term strategies to control HLB in the face

of a changing, adapting pathogen. In order to accomplish those goals, four major objectives have been developed, as outlined directly below. The PD/CoPDs involved as major contributors to efforts to accomplish each objective are indicated in parentheses. The major hypotheses underlying each objective are also listed.

Objective 1 (Beyenal, Gang, Killiny): Establish a system for in vitro culture of CLas biofilms.Hypothesis: CLas is so successful as a pathogen in citrus trees and citrus psyllids, and yet is so difficult to culture, because it grows primarily within biofilms in the psyllid and in the citrus tree. Corollaries: CLas may not be the only microbe in these biofilms and identification of the other biofilm community members may be required in order to culture CLas-containing biofilms. Synergetic interactions among the microbes in biofilms support CLas' survival.

Objective 2 (Omsland, Gang, Killiny): Establish a system for host cell-free culture of CLas.Hypothesis: CLas is a parasitic bacterium with limited metabolic capacity that depends on scavenging of nutrients from infected tissue or surrounding cells in a biofilm for replication. By matching pathogen nutrient requirements to the organism's natural metabolic capacity, replication of CLas in pure culture will be achieved.

Objective 3 (Gang, Futch, Killiny, Brown, Beyenal and Omsland): Provide SOPs and CLas culture tools to the research community. Hypothesis: The products of this project, a CLas culturing system and information about CLas growth in vivo, will be extremely beneficial to anyone interested in HLB, and thus there will be a demand for access to such information and technology. Targeted extension activities, in addition to standard research publications, will more rapidly lead to adoption of the to-be-developed technology once it is indeed available.The original proposal outlined 3 major research objectives to be accomplished within a 5-year timeframe, plus an additional objective related to extension and outreach. One of those objectives was eliminated at the request of NIFA. For one of the major goals of the project, development of planktonic growth conditions and a planktonic culture system of CLas, we are limited by the biology of the system, where some things just take a long time to occur within the biological system. Thus, the timeframe originally proposed, where 2 - 3 years to establish conditions for host cell-free metabolic activity (i.e., not necessarily cell division) of CLas and about 5 years to develop a reliable culture system for CLas in the absence of host cells, is not likely to change much, but we are likely to be well on the way to meeting that goal by the end of the second year of the project. Indeed, we should have a good idea at that point (two year mark) how likely the project is to be a success. Thus, a two year proof of concept effort will be undertaken, which will allow enough research to provide proof of concept that the overall project is likely to be a success. If such is demonstrated, then an additional ~3 years of funding will be requested in a follow-on application.?

**APPROACH:** Methods applied to accomplish each Objective are outlined below:Objective 1 (biofilm culture method): Proof of CLas existence in biofilms in psyllids has been obtained, suggesting that identification of conditions for culture of CLas under biofilm growth are within reach with the right approach. Using methods well established by Co-PI Beyenal, CLas bacteria will be isolated from biofilms growing in insect and potentially plant tissues, and will be the subject of a battery of experiments designed to identify conditions required for their growth in in vitro biofilms using an approach that enables separation of individual microbial community members (even species found only within biofilms) and analysis of their contribution to biofilm establishment, growth, protection and metabolism. This, in combination with detailed proteomic and metabolomic analysis of the test cultures, will identify those metabolic components required for biofilm growth in vitro.Objective 2 (host cell-free and planktonic culture): Establishment of

planktonic growth will be achieved by first determining responses of CLas to nutrients for which the pathogen is a predicted auxotroph. By matching pathogen nutrient requirements to the organism's natural metabolic capacity, replication of CLas in an experimentally amenable culture system will be achieved. A major step in establishment of in vitro planktonic growth will be establishment of a cell culture model for CLas infection, which will offer i) a model to analyze interactions between CLas and insect vector tissue, and ii) a cultivation system to generate enough bacteria for physiological analysis (essential to the process of designing a host cell free cultivation system).Objective 3 (extension and outreach): The project website, scientific and extension publications and conference talks/posters, as well as cold-calls to potentially interested parties, will raise awareness of the new technology and enable the rapid dissemination of methods and developed cultures.